Commentary

Mining copper transport genes

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Copper is not only a ubiquitous metal in our modern, technological environment, it is also essential for the function of most living organisms. Just as it allows for the movement of electrons through wires, it helps catalyze the movement of electrons within biological molecules. Making up only 0.01% of the Earth's crust, it is relatively scarce in the environment and must be actively scavenged. Until recently, little was known about how trace amounts of dietary copper are assimilated by intestinal absorptive cells to enter the body. In this issue of PNAS, three groups report genetic studies that may give insight into this process. Kuo *et al.* (1) and Lee *et al.* (2) report targeted gene disruption of a putative copper uptake molecule, Ctr1. In an accompanying report, Hamza and colleagues (3) report disruption of the gene encoding a copper chaperone, which escorts copper to sites of use and export within the cell.

The story begins in yeast. For copper, more than any other essential metal, *Saccharomyces cerevisiae* has proven to be a *model* model system. Ironically, insights into copper metabolism initially came from experiments aimed at understanding how cells take up iron. Dancis, Kaplan, Klausner, and colleagues (4, 5) set up genetic screens to find mutations that protected yeast cells from excess environmental iron, hoping to identify components of the high-affinity iron uptake system. One of the first genes they found encoded a putative transmembrane transport protein that, to their surprise, had no affinity for iron, but rather transported copper. This protein, designated Ctr1p, supplies the metal for a multicopper ferroxidase needed for high affinity iron transport.

Until that time, little was known about how copper enters eukaryotic cells, although some details of copper export had been elucidated through studies of human patients with Menkes disease and Wilson disease (to be discussed later). But investigators were quick to exploit the yeast data. Using Ctr1p-deficient yeast cells as living test tubes, plant (6), human (7), and mouse (8) cDNAs were isolated that rescued their copper-deficient phenotype. Gratifyingly, in each case the cDNAs encoded proteins with homology to Ctr1p. Transfection studies showed that the mammalian homologs also could stimulate copper accumulation in animal cells (2, 9). Although their normal subcellular localization has not been described, the transfection results suggest that they are expressed on the cell surface.

These data indicated that mammalian CTR1 proteins could act as copper transporters, but did not prove that they serve that function *in vivo* in their normal context. To investigate that question, two laboratories (1, 2) undertook targeted gene disruption of murine *Ctr1*. The results of the two knockout experiments are similar, but not quite identical.

Both groups replaced all four exons of the compact *Ctr1* gene with a neomycin selection cassette. They transmitted the disrupted allele through the mouse germ line and analyzed heterozygous mice for copper content. Intriguingly, tissue copper levels were about half normal in spleen (2) and brain (1, 2), consistent with a role for Ctr1 either in intestinal uptake of copper or entry of copper into those tissues. The heterozygotes were otherwise indistinguishable from wild-type mice. In contrast, the phenotype of homozygous *Ctr1*−/− mice was dramatic. They invariably died *in utero*, but the timing of embryonic demise differed slightly between the two experiments. Kuo *et al.* (1) found no remnants of *Ctr1*−/− embryos after embryonic day 9.5 (E9.5). Severe developmental defects were detectable as early as E6.5. In contrast, Lee *et al.* (2) found *Ctr1*−/− embryos in Mendelian proportions as late as E10.5. In both cases, *Ctr1*−/− embryos were highly abnormal, with severe growth retardation dating to E6.5–E7.5, impaired gastrulation, developmental defects in both neural ectodermal and mesodermal cell layers, and diminished mesenchymal cell formation and/or migration.

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These results indicate that Ctr1 plays an essential role early in embryogenesis. This is surprising if one interprets the data to conclude that copper is limiting for a very early function. As discussed below, the known copper-containing enzymes have highly specialized functions, and none is an obvious candidate for the cause of embryonic lethality, although Kuo *et al.* (1) have speculated that lysyl oxidase might be important for formation of embryonic structures.

There are at least two alternative hypotheses that should be entertained. First, both groups modified the murine *Ctr1* locus by replacement of the gene with a new transcriptional unit. It is plausible that the embryonic lethality is not a result of loss of Ctr1, but rather the result of altered expression of another nearby gene. Lee *et al.* (2) mention that they have rescued the knockout animals through transgenic expression of Ctr1, but details are not provided. The possibility that there is alteration of expression of a critical nearby gene could be eliminated by retargeting of the *Ctr1* locus with an excisable selection marker. However, the expression pattern of the *Xenopus* homolog, Xem1, argues that that experiment is probably unnecessary (10).

Alternatively, although murine Ctr1 certainly can act as a copper transporter, it remains possible that it also transports another metal, such as nickel or zinc, both neighbors to copper in the periodic table. This is not far-fetched—zinc is known to compete with copper for intestinal absorption. Furthermore, the mammalian iron transporter DMT1 (also known as Nramp2, DCT1) and the *Arabidopsis* iron transporter IRT1 both have been shown to be promiscuous in their transport of heavy metal ions (11–13). It is unclear whether mammals require nickel for cellular metabolism, but they certainly require zinc, and relatively little is known about how zinc enters cells. It might be, for example, that zinc is required at an early develop-

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mental time when Ctr1 is the only uptake system available.

If the early lethality is due to copper deficiency, it is reasonable to conclude that Ctr1 is the exclusive transporter responsible for mammalian copper uptake, at least early in embryogenesis. This would be an important distinction between unicellular and multicellular eukaryotes, because yeast has redundancy in its copper uptake genes. In addition to *CTR1*, *S. cerevisiae* has a second high-affinity copper transporter gene, *CTR3* (which is interrupted by a transposon in many lab strains; ref. 14), and a low-affinity transporter gene, *CTR2*. In humans (and probably also in mice), a CTR-related homolog is located close to *CTR1* in the genome, but its function remains uncertain (7). Obviously, neither the mammalian CTR homolog nor DMT1, which is capable of copper transport in an oocyte assay (11), can substitute for Ctr1 activity in the *Ctr1*$-/-$ mice.

The severity of the *Ctr1*$-/-$ phenotype is striking when compared with that of the microcytic anemia (*mk*) mouse, which carries a severe loss-of-function mutation in the iron transporter DMT1 (15). Like Ctr1, DMT1 transports metal into cells. Although not a total knockout, the DMT1 protein from homozygous *mk* mutants has little residual iron transport activity (16) and fails to localize properly in intestinal absorptive cells (17). The normal body iron endowment is much larger than its copper endowment, and iron is arguably more important in mammalian biology. Like copper, iron is involved in various redox reactions, but it is also essential for hemoglobin, where it serves in the transport of oxygen. DMT1 is the only molecule that has been proven to act directly in transmembrane iron entry into mammalian cells. However, *mk*/*mk* mice often survive past birth, and some live into adulthood. Somehow, small amounts of iron do get into cells. It is intriguing that the same may not be true for copper in animals lacking Ctr1.

Although experimental proof is not yet available, it seems reasonable to speculate that Ctr1 may be the intestinal portal of entry for dietary copper. Obviously, validation of this hypothesis will require studies of the expression and localization of Ctr1 in intestinal epithelial cells and probably also the production of genetically modified mice with tissue-specific loss of Ctr1 function. Assuming that dietary copper is taken up by Ctr1, one might expect less severe mutations in the protein to result in copper deficiency rather than total embryonic lethality. Intriguingly, a family has been described with inherited hypocupremia, decreased activity of copper-dependent enzymes, and clinical response to increased copper intake (18). However, biochemical studies using fibroblasts from two affected family members suggested that cellular copper accumulation is increased, rather than decreased as was expected (19).

An alternative candidate for the defect responsible for familial hypocupremia (18) might be proposed based on a report from Hamza and colleagues (3), also in this issue of PNAS, describing targeted disruption of the murine *Atox1* gene. However, to understand the function of Atox1, it is necessary to consider not copper uptake, but copper egress from cells.

Two copper export proteins initially were discovered through identification of the genes responsible for human diseases with very different phenotypes. Menkes disease has features of a severe copper deficiency disorder with X-linked recessive inheritance (20). Affected infants typically have growth retardation, severe neurological impairment, mental retardation, seizures, and abnormal hair and bone abnormalities, leading to death before 5 years of age. There is a milder variant, designated X-linked cutis laxa or occipital horn syndrome, which is not lethal (reviewed in ref. 21). The gene responsible for Menkes disease, now designated *ATP7A*, was identified simultaneously by three laboratories in 1993 (22–24). It encodes a P-type ATPase (MNK) that has multiple, distinctive, copper-binding motifs near its amino terminus. Further studies of MNK showed that it also is mutated in Mottled (*Mo*) mice (25). There are many different alleles of *Mo*, also on the X chromosome, corresponding to distinct mutations in the murine MNK protein. As expected, milder alleles produce phenotypes resembling cutis laxa and occipital horn syndrome (26).

MNK is expressed in many cell types and predominantly localized to the trans-Golgi network (27). When cells are exposed to excessive copper, MNK relocates to the plasma membrane where it functions to pump copper out of the cell. Copper deficiency in patients with Menkes disease results from an inability to mobilize absorbed copper from intestinal epithelial cells. Consequently, copper fails to enter the body, and it is lost when those cells are sloughed into the gut lumen.

Later in 1993, the gene mutated in patients with Wilson disease was discovered and shown to be a close homolog of MNK (28–30). In contrast to Menkes disease, Wilson disease is a disorder of copper excess, rather than copper deficiency. Patients appear normal early in life, but over the first four decades they typically develop liver failure, basal ganglia abnormalities, and cerebral atrophy associated with copper deposition in the liver and brain (31). In this case, the mutated gene (designated *ATP7B*) encodes a P-type ATPase, WND, that is highly expressed in the liver (28–30). WND functions within the secretory apparatus, loading cytoplasmic copper onto the plasma ferroxidase ceruloplasmin, which facilitates iron export from storage cells.

Once again, there is a spontaneous mouse mutant with features similar to the human disease. Toxic milk (*tx*) mice were first identified because the offspring of homozygous mutant mothers demonstrated poor growth, experienced hypopigmentation and tremors, and died before weaning (32). This was shown to be caused by a lack of copper in the mother's milk. Homozygous animals initially appeared normal, but on closer study they were found to have increased concentrations of copper in the liver and decreased ceruloplasmin and copper levels in the plasma. These abnormalities all result from mutations in the murine *Atp7b* ortholog. The gene also has been disrupted by deliberate gene targeting, to produce a mouse model of Wilson disease that lacks WND altogether (33).

S. cerevisiae also has a copper transporting P-type ATPase, designated Ccc2p. Ccc2p is homologous to MNK and WND and serves a similar function in transporting cytoplasmic copper into an endosomal compartment for incorporation into a ceruloplasmin homolog bound for the cell surface (34). The link between copper uptake by Ctr1p and copper export/utilization by Ccc2p is a small, soluble protein, Atx1p. This protein acts as a copper chaperone, escorting the potentially reactive metal ion through the cell in a protected state. Atx1p has been shown to interact with the amino terminal copper-binding domain of Ccc2p in two-hybrid association studies (35). Its human homolog, ATOX1 (or HAH) has been postulated to serve a similar function. ATOX1 interacts with MNK and WND proteins; in fact, some disease-causing mutations in WND disrupt this specific chaperone-transporter interaction, causing a clinical picture that is indistinguishable from Wilson disease due to mutations elsewhere in the protein (36).

Hamza and coworkers (3) report inactivation of murine *Atox1* through insertion of a drug selection/gene marker cassette into one of its introns, disrupting its transcription and abrogating protein production (3). Mice homozygous for this altered allele have a phenotype not unlike that described in the hypocupremic human family (18, 19)—they show failure to thrive with significant perinatal lethality, skin laxity, hypopigmentation, and seizures as a result of tissue copper deficiency, but their cells retain copper when assayed *in vitro*. This phenotype is similar to what might be expected from a combi-

Table 1. Copper-containing proteins and expected/known defects in their absence

Copper-containing protein	Known or expected loss of function phenotype
Ceruloplasmin	Cellular iron retention in liver and central nervous system, leading to neurodegenerative disease, iron deficiency anemia (37, 38)
Cytochrome oxidase	Metabolic acidosis, liver failure, encephalopathy, hypertrophic cardiomyopathy (39, 40)
Dopamine beta hydroxylase	Increased embryonic lethality (41), cold intolerance (42)
Factor V, factor VII	Bleeding tendency (43)
Hephaestin	Iron deficiency anemia with stainable iron in intestinal epithelial cells (44)
Lysyl oxidase	Loss of skin elasticity, hyperextensibility, vascular anomalies/aneurysms
Cu/Zn superoxide dismutase	None without further manipulations (45)

nation of the murine *Mo* and *tx* mutations. This strongly supports the conclusion that mammalian Atox1 delivers copper to P-type ATPases in much the same way as yeast Atx1p does. Interestingly, however, abrogation of expression of *Atox1* produces a less severe phenotype than some alleles of *Mo*, suggesting that it is possible for copper to find its way to the MNK transporter even when Atox1 is absent.

For the most part, the Atox1−/− phenotype approximates what might have been expected from lack of a copper transporter, even though Atox1 is not, itself, a transporter. The phenotype can be attributed to inactivity of copper-dependent enzymes (Table 1). Activities of several of these enzymes are decreased in *Atox1*−/− mice (3). This finding underscores an important concept—that nutritional deficiencies do not necessarily result from a lack of cellular uptake. Rather, they also may result from defects in cellular export, in this case consequent to the lack of an intracellular chaperone protein. To have transcellular transport, e.g., across the epithelial cell lining of the intestine, what goes in must come out.

Interestingly, *Atox1*−/− mice frequently have severe hemorrhage. Although this point was not explored in detail by Hamza *et al.* (3), one could speculate that the mice's bleeding tendency might result from decreased activity of coagulation factors V and VIII. Both of these proteins are important for normal blood clotting, contain copper, and are highly homologous to ceruloplasmin. Therefore, it is not unlikely that, like ceruloplasmin, they acquire copper through a pathway that involves Atox1. It would be interesting to explore this hypothesis further.

There are two general conclusions to be drawn from these studies. First, although serendipitous in the beginning, studies of yeast have been immensely valuable in working out the details of mammalian copper metabolism. Interestingly, this is in striking contrast to iron metabolism, where significant differences between mammals and unicellular eukaryotes have limited the utility of *S. cerevisiae* as a model system. Second, although cell biology and biochemistry are important for defining protein functions, a true understanding of mammalian metal homeostasis and diseases of metal balance can only come from *in vivo* experiments in living creatures. Our ability to manipulate the mouse genome has allowed us to understand complexities of metal deficiency and overload conditions that otherwise would be very difficult to elucidate.

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